SUPPLEMENTAL DATA:

EXPERIMENTAL PROCEDURES

Cell culture and transient transfection - HEK293 Phoenix cells were cultured in Minimum Essential Eagle Medium (MEM, Sigma, Austria) supplemented with 10% fetal bovine serum (FBS, Cambrex Bio Science), 2 mM L-glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin and 1 mM pyruvic acid (sodium salt). The mouse collecting duct M1 cells (ECACC 95092201, Sigma Aldrich, Milano, Italy) were grown in culture dishes in DMEM:Ham's F12 (1:1) base media supplemented with 2 mM glutamine, 5 µM dexamethasone, 5% FBS, 100 µg/ml penicillin, and 100 U/ml streptomycin. The cells were maintained at 37°C, 5% CO_2, 95% air and 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/EDTA treatment. For patch-clamp experiments, HEK293 Phoenix cells were seeded into Ø 30 mm Petri dishes, grown overnight and transfected with 3 µg of plasmid DNA by the calcium phosphate co-precipitation method. Eight hours post transfection, cells were seeded on glass cover slips (Ø 10 mm); electrophysiology measurements were performed 24-32 hours post-transfection. The transfection efficacy was about 50%. For patch-clamp experiments where the dimerization between HSPC038 and ICln was drug-induced, cells transfected with the suitable vectors were incubated overnight with 500 nM of the dimerizer agent AP21967 (ARGENT™ Regulated Heterodimerization Kit, Version 2.0 Ariad Pharmaceuticals, Cambridge, MA); controls cells were incubated with an appropriate amount of ethanol as the diluent (final concentration: 0.05%). For FRET experiments, M1 cells were transfected at 60-70% confluence. Transfection was carried out using polyethylenimine (PEI) reagent. Briefly, cells were incubated for 6 hours with 4 µg of plasmid DNA and 25 µl (from 1 mg/ml solution) of PEI. For double transfection with CFP-mem and YFP-HSPC038 or CFP-ICln and YFP-HSPC038, a ratio of 3:1 yielded the best results. Following transfection, cells were grown for 48 hours on uncoated cover slips. The transfection efficacy was about 15-20%. For western blot experiments, HEK293 Phoenix cells were seeded into Ø 30 mm Petri dishes, grown overnight and transfected with the selected vector (pFLAG-HSPC038, pFLAG empty, pIRES2-EGFP-EGFP, pIRES2-EGFP, or pIRES2-EGFP-HSPC038) at 60-70% confluence. Transfection was carried out using PEI reagent. Briefly, cells were incubated for 6 hours with 2 µg of plasmid DNA and 35 µl (from 1 mg/ml solution) of PEI. Following transfection, the cells were grown for 48 hours. The transfection efficacy was about 70-80%.

Patch clamp recordings - For data acquisition, an EPC-10 amplifier (HEKA Elektronik, Germany) controlled by a Macintosh computer running Patch Master (HEKA Elektronik, Germany) software was used. Access resistance as well as fast and slow capacitance were compensated and monitored throughout the recordings. All current measurements were filtered at 2.9 kHz and digitized at 2 kHz. The cells were held at 0 mV and step pulses of 400 ms duration were applied from 0 mV to 40 mV every 20 s to monitor the activation of the swelling activated chloride current (ICl_{swell}). To establish the current to voltage (IV) relationship of ICl_{swell}, step pulses of 500 ms duration were applied every 5 min from –120 mV to 100 mV in 20 mV increments from a holding potential of 0 mV. For data analysis, Fit Master (HEKA Elektronik, Germany) and EXCEL (Microsoft, USA) software were used. The current values were normalized to the membrane capacity to obtain the current density.

FRET Measurements - Detection of FRET signals in transfected cells was performed on an Olympus IX70 inverted microscope equipped with a monochromator (Polychrome 4, TILL Photonics) and a cooled charge-coupled device camera (TILL Imago SVGA) controlled by the TILL Vision software (versions 3.3 and 4.0). Measurements were performed by changing three separate Olympus BX cubes equipped with the appropriate filter combinations for ECFP, EYFP and FRET. All images were

adjusted to pixel by pixel alignment and corrected for background. netFRET calculation was performed as described by Ritter et al. (1). The integrated fluorescence density values of the images from particular regions of interest in the proximity of plasma membrane were normalized for the FRET emission determined in isotonic conditions (nFRET). Data were analyzed using TILL Vision and Microsoft Excel software. Control experiments were performed by transfecting cells with pEYFPN1 and pECFPN1 empty vectors (n=4); in these conditions, only negative FRET values were obtained (data not shown).

Protein expression and purification - The bacterial pellet obtained from a 1 liter LB culture was resuspended in 30 ml of lysis buffer (ICln and its deletion mutants: 25 mM K_2HPO_4, 150 mM NaCl, 10 mM imidazole, 5% glycerol, pH 7.2; HSPC038: 50 mM Tris, 300 mM NaCl, 5% glycerol, pH 8.0) and lysed using a French-press (2 cycles at a pressure of 12000 psi). To purify over-expressed ICln proteins, the bacterial lysate was cleared by centrifugation and loaded onto an IMAC column (HisTrap, GE Healthcare, Austria) pre-equilibrated with lysis buffer. Following extensive washing with lysis buffer, bound protein was eluted with a buffer containing 25 mM K_2HPO_4, 150 mM NaCl, 200 mM imidazole, 5% glycerol, pH 7.2. Fractions containing ICln (or its mutants) were pooled, concentrated in either Centriplus YM-30 (ICln 1-235, ICln 1-159) or Amicon Ultra 5 kDa (ICln 1-134, ICln 158-235) ultrafiltration units (Millipore, Austria) and loaded onto a Sephacryl S-100 HR column (GE Healthcare, Austria) equilibrated in 25 mM K_2HPO_4, 150 mM NaCl, 5% glycerol, pH 7.2. Fractions containing ICln (or its mutants) were collected, pooled, concentrated (see above) and stored at -70°C until use. For the purification of wild-type ICln, ICln 1-159, ICln 116-235 and ICln 135-235, IMAC was followed by anion exchange chromatography (Source Q, GE Healthcare, Austria; wash buffer: 25 mM K_2HPO_4, 150 mM NaCl, 5% glycerol, pH 7.2; elution buffer: 25 mM K_2HPO_4, 2 M NaCl, 5% glycerol, pH 7.2) prior to the final gel filtration step. For the purification of ICln 116-235, anion exchange and gel filtration chromatography were performed in the presence of 3 mM DTT. To purify HSPC038, the bacterial lysate was cleared by centrifugation and loaded onto an IMAC column (5 ml of Ni-NTA resin, Qiagen, packed into a C 10/10 column, GE Healthcare, Austria) pre-equilibrated in lysis buffer. After extensive washing with 50 mM Tris, 300 mM NaCl, 20 mM imidazole, 5% glycerol, pH 8.0, bound protein was eluted with 50 mM Tris, 300 mM NaCl, 100 mM imidazole, 5% glycerol, pH 8.0. The fractions containing HSPC038 were pooled and contaminants removed by ultrafiltration (Centriplus YM-30, Millipore, Austria). The ultrafiltrate containing HSPC038 was then concentrated (Centriplus YM-3, Millipore, Austria) and stored at -70 °C.

Western blot - Transfected cells were washed twice with ice-cold phosphate buffered solution (PBS) and incubated for 10 min on ice in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Igepal NP40 and protease inhibitor cocktail (Roche). Subsequently, cellular debris was pelleted by centrifugation at 16000 *x g* for 30 min at 4°C and the supernatant was collected. Typically, 10 µg of total proteins were separated on a 13% SDS-polyacrylamide gel and electroblotted on a PVDF membrane (Bio-Rad). Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk diluted in TBS (NaCl 150 mM, Tris-HCl 15 mM) containing 0.1% Tween (TBST). To detect ICln and tubulin, anti-ICln (2) and anti-α-tubulin (Millipore) antibodies were used at 1:1000 and 1:5000 dilutions, respectively, in blocking buffer. After washing 3 times in TBST, membranes were incubated for 1 hour at room temperature with a horseradish peroxidase (HRP) conjugated anti-rabbit antibody (Pierce) for ICln and an HRP-conjugated anti-mouse antibody for α-tubulin (Pierce). Membranes were then washed 3 times in TBST, and chemiluminescence detection was performed using an enhanced chemiluminescence kit according to the manufacturer's protocol (Pierce). Specific bands were visualized with a BioSpectrum 500 imaging system (UVP, UK). VisionWorksLS software (version 6.3.3, UVP, UK) was used for the analysis of chemiluminescent signals. For preparation of membrane proteins, transfected cells were incubated on the cell culture plate for 0, 5, 15, and 30 min in hypotonic buffer (in mM: NaCl 90, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 5, HEPES 10, pH 7.4). Following hypotonic treatment, cells were lysed and membranes were extracted as described previously (3). After resuspending total membrane fractions in PBS with Igepal CA 630 (Sigma), the protein concentration was determined using the amido black assay (4). Samples were electrophoresed and prepared for immunodetection as described above. Primary antibodies included anti-ICln, anti-FLAG

M2 (Sigma) and anti-Na^+/K^+-ATPase (Upstate). Anti-FLAG M2 was used at 1:2000 and anti-Na^+/K^+-ATPase was used at 1:10000.

REFERENCES

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4. Kaplan, R. S., and Pedersen, P. L. (1985) *Anal Biochem* **150**, 97-104

FIGURE LEGENDS

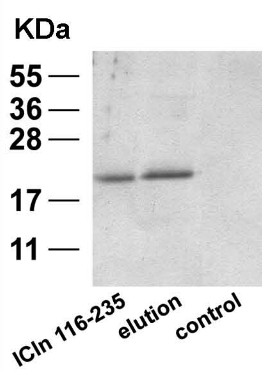
Figure S1 – Molecular interaction between ICln and HSPC038. Affinity chromatography eluates separated by SDS-PAGE of **(a)** ICln 116-235 and **(b)** ICln 135-235. For each panel, lane 1: input; lane 2: elution and lane 3: control (i.e. the elution fraction from beads with no HSPC038 bound to the resin). The presence of a band in the elution fraction indicates that ICln truncation mutants interacted with HSPC038. $2 \leq n \leq 4$. (**c**) summary of the results of the affinity chromatography experiments; ** denotes a strong interaction, * denotes a weak interaction; the absence of asterisk indicates that no interaction occurred.

Figure S2 - Effects of HSPC038 on membrane associated ICln. (**a**) Western blots showing the endogenous expression of ICln and Na^+/K^+-ATPase in total membrane extracts from cells transfected with HSPC038 (FLAG HSPC038) or with the empty vector (FLAG empty) in isotonic conditions (0 min) or after 5, 15 and 30 min of hypotonic shock. (**b**) densitometry of the ICln signal normalized to the Na^+/K^+-ATPase signal; data were collected from 20 independent samples; *** = $p<0.001$, two-way ANOVA.

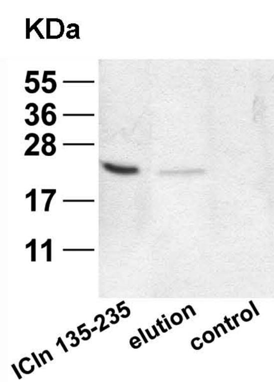
Figure S3 - Effects of HSPC038 on endogenous ICln expression. (**a**) Western blots showing the endogenous expression of ICln and tubulin in cells transfected with HSPC038 (pIRES2-HSPC038-EGFP) or with control vectors (pIRES2-EGFP-EGFP, pIRES2-EGFP) in total cell lysates. For each condition the blot of two independent samples (1 and 2) is shown. (**b**) densitometry of the ICln signal normalized to the tubulin signal; n indicates the number of independent samples. No significant differences between the data were detected (one-way ANOVA).

Fig. S1

a.



b.



c.

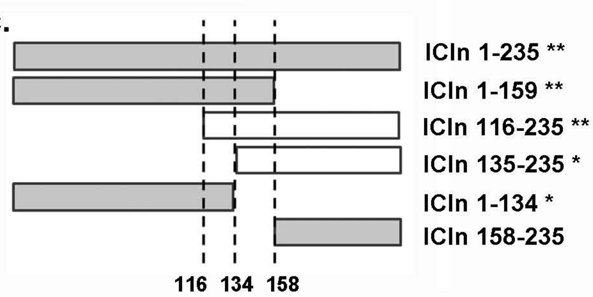
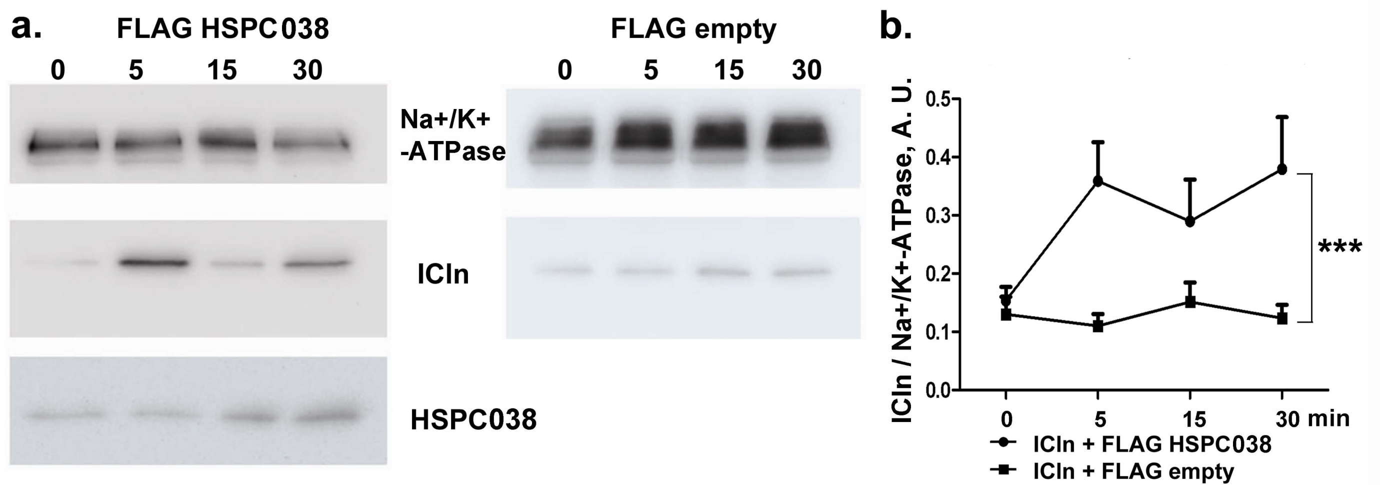


Fig. S2



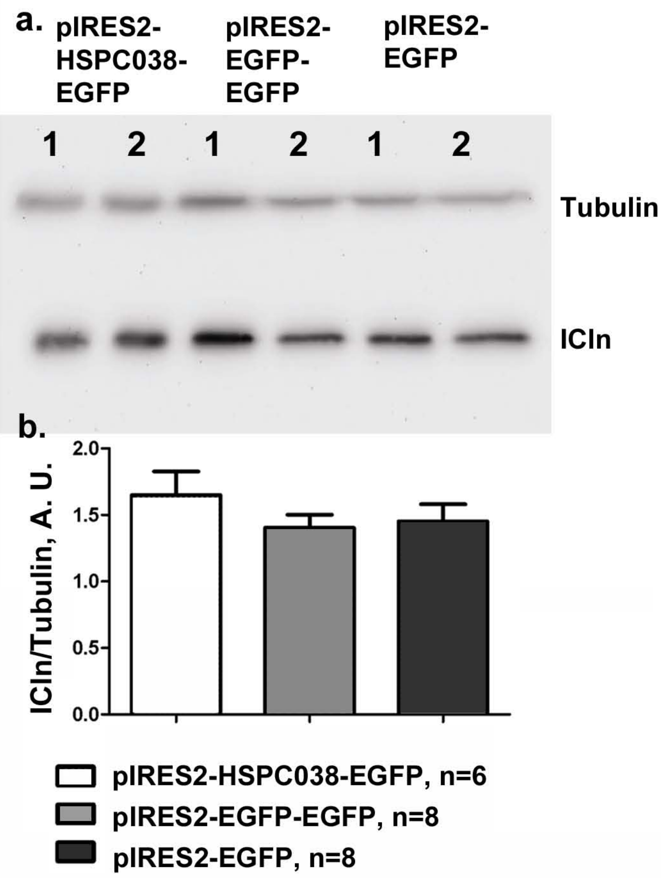


Fig. S3
a.
pIRES2-
HSPC038-
EGFP
pIRES2-
EGFP-
EGFP
pIRES2-
EGFP
1
2
1
2
1
2
Tubulin
ICln
b.
ICln/Tubulin, A. U.
2.0
1.5
1.0
0.5
0.0
pIRES2-HSPC038-EGFP, n=6
pIRES2-EGFP-EGFP, n=8
pIRES2-EGFP, n=8